

AMENDMENTS TO THE SPECIFICATIONIN THE SPECIFICATION:

Please insert the paper copy of the Sequence Listing enclosed herewith immediately after the abstract.

Please amend the specification as follows:

Please amend paragraph [0005] at page 2, lines 3-14 as follows:

B1
The GFP excitation spectrum shows an absorption band (blue light) maximally at 395 nm with a minor peak at 470 nm, and an emission peak (green light) at 509 nm. The longer-wavelength excitation peak has greater photostability ~~then~~ than the shorter peak, but is relatively low in amplitude (Chalfie et al., 1994, *Science*, 263: 802-805). The crystal structure of the protein and of several point mutants has been solved (Ormo et al., 1996, *Science* 273, 1392; Yang et al., *Nature Biotechnol.* 14, 1246). The fluorophore, consisting of a tripeptide at residues 65-67, is buried inside a relatively rigid beta-can structure, where it is almost completely protected from solvent access. The GFP absorption bands and emission peak arise from an internal p-hydroxybenzylideneimidazolidinone chromophore, which is

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ant
generated by cyclization and oxidation of the tripeptide
sequence Ser-Tyr-Gly sequence at residues 65-67 (Cody et al.,
1993, *Biochemistry* 32: 1212-1218).

Please amend paragraph [0009] at page 4, lines 11-20 as
follows:

B2
The dissection and subsequent reassembly of a protein from
peptidic fragments ~~provides~~ provide an avenue for controlling
its tertiary structure and hence its function. Although a
majority of leucine zippers associate in a parallel fashion,
recent examples of both naturally occurring and designed
antiparallel leucine zippers have appeared in the literature
(Lupas, A., 1996, *Trends Biochem. Sc.* 21, 375-382; Kohn, W. D.
et al., 1997, *S. J. Biol. Chem.* 272, 2583-2586; Bryson, J. W. et
al., 1995, *Science*, 270, 935-941; Oakley M. G. et al., 1998,
Biochemistry, 37, 12603-12610, Oakley, M. G. et al., 1997,
Biochemistry, 36, 2544-2548). However, the prior art does not
disclose the attachment of antiparallel leucine zippers to
polypeptide fragments to form fusion proteins for reassembling
the polypeptide fragments into functional proteins.

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Please amend paragraph [0012] at page 5, lines 10-17 as
follows:

B3 The association and dissociation of proteins ~~is~~ are crucial to all aspects of cell function. Examples of protein-protein interactions are evident in hormones and their respective receptors, in intracellular and extracellular signalling events mediated by proteins, in enzyme substrate interactions, in intracellular protein trafficking, in the formation of complex structures like ribosomes, viral coat proteins, and filaments, and in antigen-antibody interactions. Intracellular assays for detection of protein interactions and identification of their inhibitors have received wide attention with the completion of the human genome sequence.

Please amend paragraph [0033] at page 11, line 26 to page 12, line 2 as follows:

B4 **Figure 4** shows the antiparallel leucine zipper pairs attached to CGFP and NGFP ~~are shown~~ in helical wheel representations. The Lys (K) residues are colored blue and complementary Glu (E) residues are colored red. The pairs a and b are electrostatically matched and the pairs c and d are electrostatically mismatched. The inset shows restreaks of single *Escherichia coli* colonies corresponding to each pair. EK-CGFP is the same as CZGFP, and EK-NGFP is the same as NZGFP.

Please amend paragraph [0036] at page 12, lines 19-21 as follows:

BS The present invention is based on the finding that the dissection and subsequent reassembly of a protein from peptidic fragments ~~provides~~ provide an avenue for controlling the protein's tertiary structure and hence its function.

Please amend paragraph [0047] at page 14, lines 18-22 as follows:

Bl As used herein, "random peptide library" or a "combinatorial library" refers a library comprising not only of a set of recombinant DNA vectors (also called recombinants) that encodes a set of random peptides, but also of random peptides encoded by those vectors, as well as the fusion proteins containing those random peptides.

Please amend paragraph [0049] at page 15, lines 4-19 as follows:

B7 The present invention is based in part on the use of GFP as model for protein reassembly and fragment complementation based assays. GFP provides an ideal system for these assays because the reassembled protein autofluoresces and is easily visualized and amenable to fluorescence activated cell sorting (Tsien, R. Y., 1998, *Annu. Rev. Biochem.*, 67, 509-544; Misteli, T. et al.,

1997, *Nat. Biotechnol.* 15, 961-964). GFP fluorescence does not require the addition of other cellular factors, substrates, or additional gene products from *A. victoria*. Moreover, GFP can be expressed and detected in various cells and organisms and is not localized to a specific organelle of a cell upon expression.

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cmf
Additionally, unlike the DHFR assay, detection of GFP expression is not dependent upon survival or death of host cells. Nor is the expression of GFP dependent upon the addition of cofactors as in the β -galactoside assay or of other cellular components as in the ubiquitin assay. It is also not toxic to mammals and has been expressed in monkeys (Chan et al., 2001, *Science*, 291, 309). Further, the multiple variants of GFP available for use in different organisms and cell-types ~~makes~~ make it an ideal protein candidate for development of a general assay such as the GRIP assay described below.

Please amend paragraph [0054] at page 16, line 28 to page 17, line 9 as follows:

B8
The present invention is also based in part on the discovery that an antiparallel leucine zipper is useful for *in vivo* reassembly of protein fragments into a functionally active protein. Specifically, equimolar ~~amount~~ amounts of plasmids encoding NZGFP and CZGFP were transformed into *E. coli* cells. Colonies that turned green (Figure 3, panel a) were selected and

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further cultured in liquid media for analysis of the protein expression pattern. As shown in figure 3, panels b and c, the green colonies expressed similar amounts of NZGFP and CZGFP, whereas the non-fluorescent colonies contained either NZGFP or CZGFP. Moreover, control cotransformation experiments with NGFP/CGFP, NGFP/CAFP, and NZFP/CGFP did not have any green colonies. Accordingly, the presence of both NZ and CZ leucine zippers are required to mediate GFP assembly *in vivo* and *in vitro*.

Please amend paragraph [0057] at page 18, lines 7-20 as follows:

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Specifically, the inventors having established that the GRIP assay was selective for high affinity LZ (leucine zipper) pairs, tested the applicability of the assay in the combinatorial selection of LZ pairs that would interact strongly enough to promote GFP reassembly (Figure 5A). This would extend the GRIP system for selection of protein partners as had been demonstrated for other fragment reassembly systems (Pelletier, J. N. et al., 1999, *Nat. Biotechnol.* 17,683-690). A simple experiment in which the acidic LZ containing N-terminal GFP fragment (EE-NGFP) was kept constant was chosen. A library of LZ partners that could either code for Glu or Lys with equal probability at the e and g "specificity" positions (Figure 5A)

was generated. This library was fused to the C-terminal GFP fragment (XX-CGFP). The plasmid encoded library of XX-CGFP and EE-NGFP were cotransformed into host cells, and colonies that exhibited fluorescence were selected and analyzed by sequencing. As expected, there was an overall enrichment of Lys residues as the selected ~~partner~~ partner for complementing the acidic EE-NGFP. The electrostatic pairing of Lys/Glu is required for stabilizing the leucine zipper.

Please amend paragraph [0067] at page 20, lines 23-29 as follows:

This assay may also be used to investigate libraries of DNA, RNA, carbohydrates, peptides or other small molecules. In this situation "X-Y" can be a library. "X" is held constant with a known DNA, RNA, carbohydrate, or small molecule that binds a protein, "A", and "Y" can be varied as desired. The fusion proteins A-NGFP and Z-CGFP can also be held constant. "Y" is ~~to~~ identified and is a molecule that binds Z-CGFP. Establishing fluorescence will indicate identification of a DNA, RNA, carbohydrates, or small molecules component Y that binds protein Z.

Please amend paragraph [0069] at page 21, line 9 to page 22, line 2 as follows:

B11
Preparation and screening of combinatorial chemical libraries ~~is~~ are well known to persons of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (see, e.g., U.S. Pat. No. 5,010,175, Furka, 1991, *Int. J. Pept. Prot. Res.* 37, 487) and Houghton et al., 1991, *Nature* 354, 84). Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptoids (PCT Publication No. WO 91/19735), encoded peptides (PCT Publication WO 93/20242), random bio-oligomers (PCT Publication No. WO 92/00091), benzodiazepines (U. S. Pat. No. 5,288,514), diversomers such as hydantoins, ~~benzodiazepines and dipeptides~~ benzodiazepines and dipeptides (Hobbs et al., 1993, *Proc. Nat. Acad. Sci. USA* 90, 6909), vinylogous polypeptides (Hagihara et al., 1992, *J. Amer. Chem. Soc.* 114, 6568), nonpeptidal peptidomimetics with β -D-glucose scaffolding (Hirschmann et al., 1992, *J. Amer. Chem. Soc.* 114, 9217), analogous organic syntheses of small compound libraries (Chen et al., 1994, *J. Amer. Chem. Soc.* 116, 2661), oligocarbamates (Cho et al., 1993, *Science* 261, 1303), and/or peptidyl phosphonates (Campbell et al., 1994, *J. Org. Chem.* 59, 658), nucleic acid libraries, peptide nucleic acid libraries (U. S. Pat. No. 5,539,083), antibody libraries (Vaughn et al., 1996, *Nature Biotechnology* 14(3), 309 and PCT/US96/10287), carbohydrate libraries (Liang et al., 1996, *Science* 274, 1520

and U.S. Pat. No. 5,593,853), small organic molecule libraries (benzodiazepines, Baum, C&EN January 18, page 33 (1993); isoprenoids, U.S. Pat. No. 5,569,588; thiazolidinones and metathiazanones, U.S. Pat. No. 5,549,974; pyrrolidines, U.S. Pat. Nos. 5,525,735 and 5,519,134; morpholino compounds, U.S. Pat. No. 5,506,337; benzodiazepines, U.S. Pat. No. 5,288,514, and the like).

Please amend paragraph [0071] at page 22, lines 10-13 as follows:

The small molecules of a small molecule combinatorial library may be selected from at least one of the group consisting of amino acids, peptides, oligonucleotides, and heterocyclic compounds. The present invention contemplates ~~contemplates~~ combinatorial libraries of small molecules that are naturally occurring or synthetic.

Please amend paragraph [0074] at page 23, lines 6-14 as follows:

Suitable heterocyclic compounds consist of, at minimum, a single four membered ring to as much as a multiple of four membered or greater membered rings coupled by carbon chains of 1 to about 20 atoms in length, such chains being saturated or not. Preferably, suitable heterocyclic compounds include a single

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four- to seven-membered ring, as well as, but not limited to varying combinations of 5, 6, or 7 membered rings having varying numbers of N, S, or O atoms. Examples of suitable heterocyclic compounds include benzodiazepine and derivatives thereof (as, for example, disclosed in Bunin et al., 1992, *J. Am. Chem. Soc.* 114, 10997), ~~penicillins~~, penicillins, cephalosporins, and folate derivatives.

Please amend paragraph [0083] at page 25, lines 22-28 as follows:

P14
In a preferred embodiment, the bias is toward peptides that interact with the known classes of molecules. For example, it is known that SH-3 peptides bind ~~binds~~ to SH-3 proteins. A large number of small molecule domains are known that are suitable as starting points for the generation of biased randomized peptides. Examples of such molecules, domains, or consensus sequences include, but are not limited to SH-2 domains, SH-3 domains, pleckstrin, death domains, protease cleavage/recognition sites, enzyme inhibitors, enzyme substrates, and Traf., and leucine zipper consensus sequence.

Please amend paragraph [0094] at page 28, lines 15-25 as follows:

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Constructs for Library Selection: For leucine zipper library construction, two overlapping degenerate oligonucleotides containing NAG (N= G or A) at all positions corresponding to Lys in the leucine zipper of KK-CGFP were synthesized such that they would code for either Lys or Glu with equal probability. The two overlapping oligonucleotides were mutually primed and extended using T7 Sequenase (Amersham) with 10 mM dNTPs. The product was purified from an agarose gel and subsequently ligated into the NheI-DraIII (New England Biolabs) cassette present in a previously cut KK-CGFP plasmid. The resulting library, XX-CGFP, was transformed in 5 x 50 μ L of electrocompetent XL1-Blue cells (Stratagene) and selected for ampicillin resistance. The resulting pool of XX-CGFP plasmids was sequenced to verify that G/A were equally represented at sites of randomization.

Please amend paragraph [0095] at page 28, line 27 to page 29, line 22 as follows:

B14
Colorometric Selection: For all reassembly experiments with NZGFP/CZGFP, NZGFP/KK-CGFP, EE-NGFP/CZGFP, and EE-NGFP/KK-CGFP: 1 μ g of each plasmid was cotransformed in 30 μ L of BL21 (DE3) cells and selected on ampicillin containing LB plates. The plates were incubated at 37 °C overnight and subsequently moved

to the bench top (23 °C) for 2 ~~day~~ days. The green color developed after 16-32 hours. The cotransformation efficiency was approximately $7 \pm 2 \%$ as verified by growing up individual

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cont colonies and monitoring protein expression profiles, which corresponded well with visual inspection of green colonies in experiments with NZGFP/CZGFP and EE-NGFP/KK-NGFP. Non-fluorescent colonies that coexpressed either NZGFP/KK-CGFP or EE-NGFP/CZGFP were identified by screening 120 colonies of respective cotransformations by SDS gel for protein expression of both gene products. In library selections, 20 individual cotransformations of 1 μ g of XX-CGFP library plasmid with 1 μ g of EE-NGFP plasmid were carried out as described above. Sixteen colonies were selected from 102 green colonies of ~4000 total colonies. The colonies were grown overnight in LB media and the plasmid DNA (XX-CGFP + EE-NGFP) purified and sequenced using primers unique to the XX-CGFP construct.

Inhibition of Protein-Protein Interactions: The protein products for NZGFP, CZGFP, NGFP and CGFP were overexpressed in BL21 (DE3) cells at 37 °C and purified as described above. Amino acid analysis of the proteins established the correct compositions and protein concentrations for fluorescence experiments. The inhibitor peptide corresponding to the leucine zipper of NZGFP (EK peptide) having the sequence ALKKELQANKKELAQLKWELQALKKELAQ

(SEQ ID NO: 1) was synthesized at the Keck facility (Yale University) and purified on a reverse phase C8 column (Vydac) by HPLC. Peptide concentrations were determined by Trp absorbance and verified by amino acid analysis.

Please amend paragraph [0098] at page 30, lines 18-24 as follows:

The variant GFP (sgl00) was dissected at a surface loop between residues 157 and 158, a position that has previously been shown to accommodate a 20 residue amino acid insertion (Abedi, M. R., et al., 1998, Nucleic Acid Res., 26, 623-630). (Abedi, M. R., et al., 1998, Nucleic Acid Res., 26, 623-630). The dissection resulted in N- and C-terminal fragments, designated NGFP and CGFP, containing 157 and 81 residues, respectively (Figure 1). The NGFP fragment contains the three residues, Ser65, Tyr66, and Gly67, that ultimately form the GFP fluorophore (Tsien, R. Y., 1998, Annu. Rev. Biochem., 67, 509-544).

Please amend paragraph [0117] at page 36, lines 19-21 as follows:

Examples of other macromolecular interactions include, but are not limited to, nucleic acid-nucleic acid binding protein interactions and carbohydrate-protein interactions.